MECHANISMS UNDERLYING SKELETAL MUSCLE WEAKNESS
IN HUMAN HEART FAILURE:
ALTERATIONS IN SINGLE FIBER MYOSIN PROTEIN CONTENT AND FUNCTION

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ABSTRACT

Background: Patients suffering from chronic heart failure frequently experience skeletal muscle weakness, which limits physical function. The mechanisms underlying muscle weakness, however, have not been clearly defined.

Methods and Results: The present study examined the hypothesis that heart failure promotes a loss of myosin protein from single skeletal muscle fibers, which in turn reduces contractile performance. Ten patients with chronic heart failure and 10 controls were studied. Muscle atrophy was not evident in patients, and groups displayed similar physical activity levels, suggesting that observed differences reflect the effects of heart failure, not muscle atrophy or disuse. In single muscle fibers, heart failure patients showed reduced myosin heavy chain (MHC) protein content (P-values: <0.05) that manifested as a reduction in functional myosin-actin cross-bridges (P<0.05). No evidence was found for a generalized loss of myofilament protein, suggesting a selective loss of myosin. Accordingly, single muscle fiber maximal Ca\textsuperscript{2+}-activated tension was reduced in patients in MHC I fibers (P<0.05). Tension was maintained in MHC IIA fibers in patients, however, because a greater proportion of available myosin heads were bound to actin during Ca\textsuperscript{2+}-activation (P<0.01).

Conclusions: Collectively, our results show that heart failure alters the quantity and functionality of the myosin molecule in skeletal muscle, leading to reduced tension in MHC I fibers. Loss of single fiber myosin protein content represents a potential molecular mechanism underlying muscle weakness and exercise limitation in heart failure patients.

Key Words: heart failure, skeletal muscle, mechanics, myosin
INTRODUCTION

Patients suffering from chronic heart failure have a reduced capacity for physical work. Although cardiac dysfunction is the primary pathological insult, the resulting syndrome of heart failure alters numerous physiological systems to impair functional capacity. Alterations in skeletal muscle are of particular importance, most notably atrophy, weakness, and reduced endurance. These peripheral skeletal muscle adaptations limit physical function independent of cardiac impairment and persist despite correction of cardiac dysfunction.

Aerobic fitness is commonly assumed to be the primary determinant of physical function in heart failure patients because exertional fatigue and dyspnea are the predominant symptoms of the disease. However, aerobic capacity is a relatively poor predictor of performance in activities of daily living. This is because most activities are limited instead by skeletal muscle strength. Accordingly, functional capacity can be increased in heart failure patients in the absence of alterations in aerobic capacity by improving muscle strength. Despite its potential relevance to physical disability in patients with heart failure, few studies have explored the mechanisms underlying reduced skeletal muscle strength.

Skeletal muscle weakness in heart failure is not explained by muscle atrophy or reduced motor activation, suggesting defects in the intrinsic contractile properties of individual muscle fibers. Recent studies in rats and humans in chemically-skinned single muscle fibers have shown reduced contractile function in heart failure, implicating impaired myofilament protein function in muscle weakness. Because myosin is the most prevalent myofilament protein in muscle and is the primary determinant of single fiber contractile mechanics, alterations in fiber function can be linked to variation in the quantity or function of the myosin molecule. In this context, our previous work demonstrating a reduction in myosin heavy chain (MHC) protein content in skeletal muscles of heart failure patients suggests that contractile dysfunction may result from a reduced quantity of myosin. Indeed, a recent study in rats with heart failure has shown reduced MHC protein content and force production in single diaphragm muscle fibers.
Additionally, this study found alterations in myosin kinetic properties that could diminish contractile function.\textsuperscript{11} Whether similar alterations in the quantity and functionality of myosin occur in skeletal muscle of patients with heart failure, however, has not been examined.

The goal of this study was to examine the effect of heart failure on single skeletal muscle fiber myofilament protein composition, function and sarcomeric structure. To accomplish this objective, we evaluated single muscle fibers from the vastus lateralis muscle of patients with chronic heart failure and sedentary controls. Controls were recruited to match the low physical activity levels typically observed in heart failure patients,\textsuperscript{16} which obviates the effects of muscle activity level on single fiber structure and function.\textsuperscript{17}
METHODS

Subjects. Ten patients (7 men, 3 women) with physician-diagnosed heart failure were recruited. The population consisted of patients with both systolic dysfunction (left ventricular ejection fraction (EF) <40%; n=6; 26.0 ± 2.7%; range: 17-35%) and preserved systolic function (EF>40%; n=4; 46.8 ± 1.8%; range: 45-52%). At the time of study, there were 1 NYHA class I, 5 class II and 4 class III patients. The etiology of heart failure was ischemic in 3 volunteers and non-ischemic in 7. Three patients had Type II diabetes mellitus. All patients were clinically stable and had not been hospitalized for at least 6 months prior to testing. None had evidence of hepatic, renal, or peripheral vascular disease or an active neoplastic process. Patients were receiving angiotensin-converting enzyme (ACE) inhibitors/receptor blockers (100%), β-blockers (90%), diuretics (70%), HMG CoA reductase inhibitors (20%) and one female patient was receiving levothyroxine. Plasma creatine kinase levels were normal in all patients and none were smokers or taking sex steroid replacement therapy.

Controls (n=10; 6 men, 4 women) were recruited who were sedentary or minimally-active, as defined by self-report of ≤2 sessions of ≥30 min of exercise/week and not participating in any exercise training. Controls were non-smokers, had a stable body weight (±2 kg during prior 6 months and not participating in a weight loss program), no signs or symptoms of heart failure, coronary heart disease or diabetes (fasting blood glucose >112 mg/dL), normal left ventricular ejection fraction (>55%), normal complete blood counts and routine biochemical values and were not taking sex steroid replacement therapy. Four controls had a history of hypertension and three were treated with diuretics and one with an ACE inhibitor. All were normotensive at testing and showed no evidence of left ventricular hypertrophy or atrial enlargement by echocardiography. Two controls were on stable doses of HMG CoA reductase inhibitors and one female was on levothyroxine. Plasma creatine kinase levels were normal in all controls. Informed consent was obtained from each volunteer and the protocol was approved by the
Committees on Human Research at the University of Vermont. Data showing reduced knee extensor muscle strength in heart failure patients from this cohort have been published.3

**Experimental protocol.** Eligibility was determined during screening visits, at which time medical history, physical examination, blood samples, whole muscle strength testing, a treadmill test and echocardiography were performed. At least 1 week later, eligible volunteers underwent an inpatient visit. Prior to admission, medications were maintained per normal dosing regimens, except coumadin (n=3), which was stopped 5 days before this visit. On the following morning, in the fasted state, body composition and mid-thigh muscle cross-sectional area was measured, and muscle tissue was obtained via percutaneous biopsy (Bergstrom needle, 5 mm OD) of the vastus lateralis.

**Total and regional body composition.** Total and regional fat mass and fat-free mass were measured by dual energy x-ray absorptiometry, as described.19 Bone mass data are not reported. Mid-thigh muscle area was measured by computed tomography, as described.18

**Peak oxygen consumption (peak VO₂).** Peak VO₂ was determined using the Naughton protocol.19

**Accelerometry.** Free-living physical activity was measured using a single-plane accelerometer, as described previously.20

**Muscle tissue processing.** Approximately two-thirds of the biopsy material was placed immediately into cold (4°C) dissecting solution (see on-line supplement for composition of solutions). Remaining tissue was frozen in liquid nitrogen and stored at -80°C. Muscle fibers were dissected into bundles and tied to glass rods at 4°C, and then placed in skinning solution
for 24 h at 4°C, storage solution with 50% (v/v) glycerol for 16 hr at 4°C and finally stored at -20°C until study (within 4 wks).

**Tissue homogenate MHC protein content and isoform distribution.** Tissue MHC protein content and isoform distribution were determined on frozen muscle tissue, as described previously, with minor modifications (see on-line supplement).

**Single muscle fiber morphology and MHC protein content.** Segments (~3 mm) of single muscle fibers were measured in relaxing solution (20°C) to estimate fiber volume (see on-line supplement for details). Aliquots (1.5 µm³ fiber volume) of sample were analyzed for MHC and actin protein content in triplicate, according to previously published methods, with modifications (see on-line supplement). Thereafter, each fiber was analyzed for fiber type via MHC isoform expression (see on-line supplement).

**Single fiber mechanical measurements.** Segments (~2.5 mm) of single fibers were isolated and their ends fixed with glutaraldehyde, as elsewhere, with modifications (see on-line supplement). Top and side diameter measurements were made in relaxing solution (pCa 8) at 3 positions to calculate cross-sectional area and the fiber was incubated in dissecting solution containing 1% Triton X-100 (v/v) for 30 min. Fibers were attached to a piezoelectric motor and a strain gauge in relaxing solution, the sarcomere length set to 2.65 µm and unfixed fiber length (~1 mm) measured. All mechanical measurements were performed at 15°C. The fiber was transferred to pre-activating solution for 30 s and then to activating solution (pCa 4.5) and tension recorded at plateau. At plateau, sinusoidal length oscillations (10 cycles of 0.125% fiber length at 250 Hz) were imposed to measure fiber dynamic stiffness. Duplicate measurements of maximal Ca²⁺-activated tension and stiffness were obtained for each fiber. Thereafter, the fiber was placed in rigor solution and, at the plateau of tension, dynamic stiffness was measured. The
amplitude of dynamic stiffness in the rigor state is proportional to the total number of available myosin heads that can bind actin (ie, total cross-bridge number), assuming all myosin heads bind to actin in rigor. The ratio of pCa 4.5 to rigor dynamic stiffness, therefore, provided an estimate of the fraction of available myosin heads that bind actin during Ca²⁺-activation. Following mechanical measurements, single fibers were analyzed for MHC isoform composition to identify fiber type.

Ultrastructural measurements. Electron microscopy measurements were conducted on intact skeletal muscle fiber bundles, as described²⁴ (see on-line supplement for details).

Protein and gene expression. Immunoblotting techniques were used to assess MHC degradation products, according to the method of Ball et al.,²⁵ and the quantity of ubiquitinated MHC (see on-line supplement). MHC isoforms, actin, muscle ring finger-1 (MuRF-1) and atrogin-1 mRNA levels were determined by real-time PCR (see on-line supplement).

Statistics. All data are reported as mean ± SE. Unpaired Student t tests were used to compare groups. Analysis of covariance was used to compare peak VO₂ data between groups after adjusting for differences in fat-free tissue mass.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
RESULTS

Groups were similar for age, body size, body mass and total and regional body composition. Peak VO₂ adjusted for fat-free mass was lower (P<0.01) in patients (Table 1). Daily physical activity level measured by accelerometry over 7.6 ± 0.4 d was similar between groups.

Heart failure patients had lower MHC protein content in tissue homogenates compared to controls (Figure 1A; P<0.01), with no group differences in the relative distribution of MHC isoforms (Figure 1B).

Fibers from patients had greater average cross-sectional area compared to controls (P<0.01; Table 2) when all fibers were pooled, with or without inclusion of those fibers with MHC band densities less than or equal to background. When partitioned into fiber types, patients had greater cross-sectional area in MHC IIA and IIA/X fibers (P<0.01 for both), but not in MHC I fibers.

Heart failure patients showed lower MHC protein content in single fibers whether fibers with MHC band densities less than or equal to background (n=39 fibers from patient group; n=25 fibers from control group) were not included (P<0.01; Figure 2A). Actin was detected in all fibers examined (see on-line supplement). In fibers with detectable MHC bands, heart failure patients had lower MHC content in MHC I (P<0.05), MHC IIA (P<0.01) and MHC IIA/X (P<0.01) fibers (Figure 2B). There were too few MHC IIX and MHC I/IIA fibers to permit comparisons.

No group differences were evident for myofibrillar volume fraction, A-band length or thick to thin filament ratio (Figure 3A-I). Average sarcomere length also did not differ (C: 2.06 ± 0.09 vs. HF: 2.20 ± 0.13 μm) and no relationship between sarcomere length and the thick to thin filament ratio was found (r=0.022; P=0.94).

Single muscle fiber Ca²⁺-activated tension and dynamic stiffness data are shown in Figure 4. Patients showed lower tension in MHC I fibers (P<0.05), while MHC IIA fibers
remained similar to controls (Figure 4A). pCa 4.5 stiffness was decreased in MHC I fibers (P<0.01) and unchanged in MHC IIA fibers (Figure 4B), while rigor stiffness was decreased in patients in both MHC I and MHC IIA (both P<0.05; Figure 4C) fibers. The pCa 4.5 / rigor stiffness ratio did not differ between groups in MHC I fibers, but was increased in patients in MHC IIA fibers (P<0.01; Figure 4D). Finally, there were a sub-set of fibers in controls (n=7) and patients (n=20) that failed catastrophically (ie, tore) upon Ca\(^{2+}\)-activation.

No differences were found between patients (n=7) and controls (n=5) in mRNA abundance for MHC I (C: 1.00 ± 0.19 vs. HF: 1.10 ± 0.33 relative expression), MHC IIA (C: 1.00 ± 0.14 vs. HF: 0.87 ± 0.09), MHC IIX (C: 1.00 ± 0.37 vs. HF: 1.68 ± 0.54) or actin (C: 1.00 ± 0.12 vs. HF: 0.96 ± 0.10). Similarly, no group differences were noted for mRNA of any of these genes when data were analyzed using 18S RNA as the housekeeping gene (see on-line supplement). Additionally, no differences between patients and controls in the amount of MHC I breakdown fragments (Figure 5A,C), ubiquitinated protein corresponding to MHC (Figure 5B,D) or expression of E3 ubiquitin ligases (Figure 5E,F) was found (see on-line supplement for further details).
DISCUSSION

In the present study, we found reduced single fiber MHC protein content in heart failure patients, which was manifest as a decreased number of functional myosin-actin cross-bridges. No evidence was found, however, for a generalized loss of myofibrillar proteins, suggesting a selective loss of myosin protein. In keeping with reduced MHC protein/cross-bridge number, single fiber maximal Ca\textsuperscript{2+}-activated tension (force per cross-sectional area) was reduced in MHC I fibers from patients. Interestingly, tension was maintained in MHC IIA fibers from patients because a greater proportion of myosin heads bound actin during Ca\textsuperscript{2+}-activation, implying altered myosin kinetic properties. Our results represent the first demonstration of an effect of heart failure on the quantity and kinetic properties of the myosin molecule in single skeletal muscle fibers in human heart failure.

Heart failure patients are profoundly inactive relative to age-matched, healthy controls,\textsuperscript{16} which complicates group comparisons because inactivity modulates muscle structure and function.\textsuperscript{26} To mitigate the effect of inactivity, we recruited sedentary controls to match patients for muscle use and confirmed that their activity levels were similar to patients. Additionally, patients were tested ≥6 months following inpatient admissions to eliminate any acute effects of muscle disuse on single fiber structure or function. An interesting observation that highlights the importance of these experimental considerations is the similar MHC isoform distribution (ie, fiber type) in patients and controls (Figure 1B). Our fiber type results agree with studies that have similarly controlled the activity status of controls,\textsuperscript{9,27} but differ from those that have not.\textsuperscript{15,28,29} In these latter studies, a shift in fiber type towards a fast-twitch phenotype was observed.\textsuperscript{15,28,29} As muscle disuse increases the number of fast-twitch fibers,\textsuperscript{30} these results collectively suggest that one of the presumed hallmark muscle adaptations to heart failure—a switch to a fast-twitch phenotype—may simply be a repercussion of the muscle disuse imposed by the disease state. Thus, experimental control for physical activity patterns is necessary to identify alterations in myofilament protein content and function that are specific to heart failure.
At the single fiber level, MHC loss was evident in all fiber types examined from heart failure patients (Figure 2B). These results extend evidence from rat models to demonstrate, for the first time, that myosin is lost from single muscle fibers as a consequence of human heart failure. Further reinforcing our MHC content data, we found a decrease in single fiber dynamic stiffness in patients at the plateau of rigor tension (Figure 4C), suggesting a loss of functional myosin heads that can bind actin to form cross-bridges. Because myosin comprises 25% of total skeletal muscle protein and 40% of myofilament protein, this reduction may simply reflect a loss of myofilament protein secondary to muscle atrophy. The fact that we found no evidence for muscle atrophy in patients, and no group differences in single fiber actin protein content (see on-line supplement) or myofibrillar volume fraction, however, argues against this conclusion and implies a selective loss of myosin protein. This type of selective myosin depletion is not unique to heart failure and has been observed in acute quadriplegic myopathy patients and a rat model of cancer, suggesting that this phenotype may be common to a variety of acute and chronic disease states.

From a structural standpoint, loss of myosin could result from a decreased number of thick filaments, shortening of the thick filaments and/or loss of myosin at random points along the thick filament. We found no evidence for a loss of thick filaments (thick-to-thin filament ratio) or shortening of the A-band (ie, thick filament) length in patients, implying a loss of myosin along the entire length of the thick filament. This structural phenotype of myosin depletion agrees with the fact that the thick filament is remodeled by replacing myosin at random points along the length of the filament.

Single fiber tension is directly related to the number of functional myosin heads, the proportion of these heads bound to actin during Ca^{2+}-activation and the force generated per cross-bridge. Thus, a loss of MHC protein would result in reduced single fiber tension. Correspondingly, tension was reduced in MHC I fibers from patients (Figure 4A). This reduction in tension is less than the decrement in MHC protein content/cross-bridge number, which may
be explained by the fact that the relationship between MHC protein content and tension appears to be non-linear.\textsuperscript{31} The non-linear nature of this relationship may relate to alterations in cross-bridge kinetics which accompany MHC protein depletion.\textsuperscript{11} Additionally, variance between single fiber MHC protein content and tension in our study may simply relate to the fact that measurements were preformed on two separate populations of fibers. Regardless of the underlying mechanisms, the diminution of function in MHC I fibers may be relevant to physical disability in heart failure patients in light of the fact that MHC I fibers are recruited for repetitive movements typically encountered in daily activities.\textsuperscript{36,37} Moreover, diminished function in MHC I fibers may contribute to reduced exercise capacity as these fibers are important determinants of aerobic fitness in heart failure patients.\textsuperscript{38} In this context, loss of myosin protein represents a potential molecular mechanism underlying physical disability and exercise intolerance in heart failure.

Interestingly, despite the reduction in MHC content, no group differences in tension were found in MHC IIA fibers. The reason for this disparity was revealed when fiber stiffness was evaluated under maximal Ca\textsuperscript{2+}-activated and rigor conditions, which demonstrated a greater proportion of myosin heads bound during Ca\textsuperscript{2+}-activation in MHC IIA fibers in patients (Figure 4D). As detailed above, a greater proportion of myosin heads bound to actin would increase tension. This adaptation could be explained by a change in myosin kinetic parameters,\textsuperscript{11} which either increases the amount of time myosin is bound to actin and/or decreases the amount of time myosin is detached from actin. Thus, our results suggest that heart failure alters both the quantity and kinetic properties of the myosin molecule in skeletal muscle, with functional consequences that are fiber-type specific.

Our current results in single fibers differ from our previous findings in isolated myosin and thin filaments evaluated using the \textit{in vitro} motility assay, which showed no effect of heart failure on force production or contractile velocity.\textsuperscript{39} These differences between studies are likely due to differences in the two assays of myofilament function. In the \textit{in vitro} motility assay, the
quantity of myosin and thin filament proteins used is standardized, which negates the functional
effect of MHC protein depletion evident in skinned fibers from heart failure patients. Moreover, in
the mixed fiber preparations used in the motility assay in our prior study, myosin kinetic
properties are dominated by MHC I molecules. Hence, alterations in MHC IIA kinetics evident
in skinned fibers (Figure 4D) would be masked in the motility assay. Additionally, our results
differ somewhat from prior studies that showed large reductions (>30%) in single fiber tension in
MHC I and IIA fibers from heart failure patients. Here again, direct comparisons between
studies are difficult since controls in this prior study were 12 yrs younger than patients and
groups were not matched for physical activity level. Thus, the large tension reductions may have
been related to aging and/or muscle disuse. In contrast, patients and controls in our study
were well-matched for age and physical activity, making our findings more reflective of the direct
effects of the heart failure syndrome.

In light of the potential relevance of MHC protein depletion, we sought to identify the
mechanisms whereby heart failure promotes a loss of skeletal muscle myosin by measuring
MHC mRNA abundance and indices of MHC protein degradation. No group differences in MHC
mRNA abundance were found, suggesting no alteration in MHC gene transcription. These
results differ from our prior work, where we observed a trend towards reduced MHC mRNA in
patients. This prior finding is likely explained by the fact that we did not match controls and
patients for physical activity. Consequently, reduced MHC mRNA was explained entirely by
decreased MHC I mRNA, which is likely due to inactivity-induced reductions in MHC I gene
expression. This further emphasizes the importance of considering the activity status of
controls. Additionally, using multiple techniques, we found no evidence for elevated MHC
protein degradation in patients (Figure 5). These results contrast with recent work in a rat model
of heart failure showing that pharmacological treatment with an inhibitor of protein breakdown
can prevent the loss of MHC from single diaphragm fibers. Reasons for differing results are
not clear, but may relate to the fact that we expect the heart failure syndrome to be more severe
and rapidly progressive in this animal model compared to well-treated, clinically-stable patients. These differences in disease status between animal models and patients highlight a potential explanation for the MHC protein content depletion observed in our study; specifically, that reduced MHC gene expression and increased proteolysis occur in patients and precipitate MHC protein loss during periods of acute disease exacerbation and hospitalization. These episodes are characterized by neurohumoral/immune activation\textsuperscript{42,43} and physical inactivity, both of which could contribute to the depletion of MHC protein.\textsuperscript{17,32}

In conclusion, our results suggest selective myosin protein depletion from individual muscle fibers as a potential molecular mechanism contributing to skeletal muscle weakness in heart failure patients. Because controls and patients were similar for age and physical activity level and there was no evidence for muscle atrophy in patients, we believe that this phenotype is reflective of the effects of the heart failure syndrome on skeletal muscle, rather than the effects of aging, muscle atrophy or physical inactivity. Moreover, the fact that these observations were made in well-treated patients with mild to moderate heart failure suggests that single muscle fiber myosin depletion is not merely a manifestation of end-stage disease, but rather a distinct feature of the skeletal muscle myopathy of heart failure.
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DISCLOSURES

No conflicts of interest to disclose.
REFERENCES


3. Toth MJ, Shaw AO, Miller MS, VanBuren P, LeWinter MM, Maughan DW, Ades PA. Reduced knee extensor function in heart failure is not explained by inactivity. *Int J Cardiol.* In Press.


failure patients versus sedentary or active control subjects. *J Am Coll Cardiol.* 2001;38:947-954.


FIGURE LEGENDS

Figure 1. Tissue homogenate MHC protein content (A; arbitrary densitometry units (AU) per µg of protein) and relative isoform distribution (B; % of total) in controls (C; n=10) and heart failure patients (HF; n=9) with representative sections of gels. Bar graphs represent mean ± SE. *, P<0.05; **, P<0.01.

Figure 2. Single fiber MHC (n=10/group) protein content from controls (C) and heart failure patients (HF) and representative gel images of MHC bands. Data are shown including all fibers measured and only those fibers that contained a measurable MHC band (density > background). The number of fibers is indicated at the base of each bar. Bar graphs represent mean ± SE. *, P<0.05; **, P<0.01.

Figure 3. Skeletal muscle fiber ultrastructural data in controls (n=8) and heart failure patients (n=7). Representative cross-sectional (8,000X; A and B; scale bar=1 µm and 60,000X; G and H; bar=100 nm) and longitudinal (5,000X; D and E; scale bar=1 µm) images are presented. Bar graphs in panels C,F and I represent mean ± SE.

Figure 4. Single skeletal muscle fiber Ca$^{2+}$-activated (pCa 4.5) tension (A) and dynamic stiffness data (B, C, D) in controls (n=5) and heart failure patients (n=9). The number of fibers is indicated at the base of each bar. Bar graphs represent mean ± SE. *, P<0.05; **, P<0.01.

Figure 5. MHC I protein breakdown fragments (A,C) and ubiquitinated MHC (B,D) in controls (C; n=4) and heart failure patients (HF; n=4) and E3 ubiquitin ligase expression (E,F; n=6 controls and 4 heart failure). Representative western blots are shown for MHC I breakdown fragments (A) and ubiquitinated MHC (B), including a Simple Blue-stained gel to indicate total MHC protein content. Bar graphs represent mean ± SE.
Table 1. Clinical characteristics and physical activity levels of controls and heart failure patients.

<table>
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<th>Controls</th>
<th>Heart failure</th>
<th>n</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>69.3 ± 4.4</td>
<td>72.2 ± 4.4</td>
<td>10/10</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.1 ± 3.3</td>
<td>170.4 ± 3.0</td>
<td>10/10</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>81.9 ± 4.0</td>
<td>92.4 ± 9.9</td>
<td>10/10</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>29.9 ± 2.3</td>
<td>32.7 ± 5.3</td>
<td>10/9</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>49.1 ± 3.3</td>
<td>51.8 ± 5.1</td>
<td>10/9</td>
</tr>
<tr>
<td>Arm fat-free mass (kg)</td>
<td>5.32 ± 0.50</td>
<td>5.27 ± 0.61</td>
<td>10/9</td>
</tr>
<tr>
<td>Leg fat-free mass (kg)</td>
<td>15.4 ± 1.8</td>
<td>14.8 ± 2.2</td>
<td>10/9</td>
</tr>
<tr>
<td>Mid-thigh muscle</td>
<td>108.8 ± 8.8</td>
<td>100.9 ± 11.2</td>
<td>8/9</td>
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<tr>
<td>cross-sectional area</td>
<td></td>
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</tr>
<tr>
<td>(cm²)</td>
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<td>Peak oxygen consumption</td>
<td>1.93 ± 0.12</td>
<td>1.24 ± 0.12 *</td>
<td>9/9</td>
</tr>
<tr>
<td>(L/min)†</td>
<td></td>
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<td></td>
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<tr>
<td>Physical activity</td>
<td>215 ± 32</td>
<td>251 ± 45</td>
<td>9/10</td>
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<tr>
<td>level (kcal/d)</td>
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Data are mean ± SE. †, peak oxygen consumption data were adjusted for fat-free mass using analysis of covariance. *, P<0.01.
Table 2. Average cross-sectional area of single skeletal muscle fibers from controls and heart failure patients.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Heart failure</th>
<th># of fibers (control/heart failure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All fibers (μm²)</td>
<td>5880 ± 145</td>
<td>6824 ± 160 *</td>
<td>194/200</td>
</tr>
<tr>
<td>Fibers with detectable MHC content (μm²)</td>
<td>5916 ± 159</td>
<td>6748 ± 169 *</td>
<td>169/161</td>
</tr>
<tr>
<td>MHC I fibers (μm²)</td>
<td>6613 ± 242</td>
<td>6538 ± 247</td>
<td></td>
</tr>
<tr>
<td>MHC IIA fibers (μm²)</td>
<td>5422 ± 210</td>
<td>7419 ± 305 *</td>
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<tr>
<td>MHC IIA/X fibers (μm²)</td>
<td>4450 ± 193</td>
<td>6091 ± 347 *</td>
<td></td>
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</tbody>
</table>

Data are mean ± SE and reflect the average cross-sectional area from top and side diameter measurements every 250 μm along the length of each fiber. Fibers with detectable MHC include only those with band densities greater than background, whereas the All fibers includes those with band densities equal to or less than background. There were too few MHC IIIX and I/IIA fibers to permit comparisons between groups. *, P<0.01.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
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MECHANISMS UNDERLYING SKELETAL MUSCLE WEAKNESS IN HUMAN HEART
FAILURE: ALTERATIONS IN SINGLE FIBER MYOSIN PROTEIN CONTENT AND KINETICS

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Supplemental Methods:

Solutions. For solutions used for Muscle tissue processing, dissection solution contained (in mM) 20 N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 5 EGTA, 5 MgATP, 1 free Mg²⁺, 1 DTT and 0.25 phosphate (Pi) with an ionic strength of 175 mEq, pH 7.0, and at pCa 8 (pCa = -log₁₀ [Ca²⁺]), skinning solution contained (in mM): 170 potassium propionate, 10 imidazole, 5 EGTA, 2.5 MgCl₂, 2.5 ATP-Na₂H₂, 0.05 leupeptin and 0.05 antipain at pH 7.0, and storage solution was identical to skinning solution, but without leupeptin and antipain. For solutions used for Single fiber mechanical measurements, calculations were performed using the equations of Fabiato and Fabiato¹ and stability constants of Godt and Lindley.² Relaxing solution was identical to dissecting solution with 15 mM creatine phosphate (CP) and 300 units/ml of creatine phosphokinase (CPK), pre-activating solution was identical to relaxing solution, except at an EGTA concentration of 0.5 mM, activating solution was the same as relaxing solution, except at pCa 4.5 and rigor solution was the same as activating solution, except that it lacked MgATP, CP and CPK. All solutions used for mechanical experiments were adjusted to proper ionic strength (175 mEq) using sodium methane sulfate. Finally,
loading buffer used for various gel electrophoresis applications was 2% SDS, 62.5 mM Tris, 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol, pH 6.8.

**Tissue homogenate MHC protein content and isoform distribution.** Muscle tissue (~20 mg) was homogenized (in mM: 250 sucrose, 2 EDTA, 10 Tris, pH 7.4), the homogenate was centrifuged (10,000 g at 4°C) for 10 min and the supernatant, which contains soluble (ie, non-myofibrillar) proteins, was decanted and the pellet resuspended in three volumes of cold (4°C) extraction buffer (in mM: 100 Na₄P₂O₇, 5 EDTA, 1 DTT, pH 8.5), incubated on ice for 30 min and then centrifuged (10,000 g at 4°C for 10 min). The supernatant, which contains myofibrillar proteins, was analyzed for protein content (BioRad; Hercules, CA). An aliquot of the supernatant was added to loading buffer (described above in Solutions section), heated for 2 min at 65°C and analyzed by SDS-PAGE.

For MHC protein content, 2 μg protein was loaded onto gels (stacking: 4% acrylamide-\(N,N'\)-methylene-bis-acrylamide (bis) and the resolving 7.5% acrylamide-bis) and run at 70V for 3 hr, which permits visualization of MHC isoforms as a single band. Gels were stained with coomassie blue, scanned and MHC band intensity determined by densitometry (Quantity One; BioRad; Hercules, CA). MHC protein content data are expressed as densitometric units per μg of protein loaded and reflect the average of duplicate measures. MHC content measurements were not performed on one patient because of lack of tissue.

The relative distribution of MHC isoforms (MHC I, IIA, IIX) was determined according to standard methods, as described previously,³ with minor modifications. Briefly, 0.2 μg protein was loaded. The stacking gel contained 4% acrylamide-bis/5% glycerol (w/v) and the resolving gel 7% acrylamide-bis/30% glycerol (w/v). Gels were run at 70V for 3.5 hr, followed by 200V for 20 hr at 9°C. This permits separation of the three
isoforms of MHC into three distinct bands. The gel was silver stained (Silver Snap;
Pierce, Rockford, IL), scanned, the MHC isoforms quantified by densitometry (Quantity
One; BioRad; Hercules, CA) and data expressed as a percentage of total MHC
densitometric units.

*single muscle fiber morphology and MHC protein content.* Fibers had T-clips were
placed at either end, were mounted in dissecting solution (20°C) under a compound
microscope, pulled taught and clip-to-clip length measured using a manual micrometer.
Using a right-angled, mirrored prism, top and side fiber diameters were measured to the
nearest tenth of a μm at 250 μm intervals along the length of the fiber at 100X using a
digital filar eyepiece micrometer (Lasico, Los Angeles, CA). Following measurements,
the fiber was cut adjacent to each T-clip, placed in loading buffer (75 μl), sonicated at
40°C for 1 hr, heated for 5 min at 65°C and stored at -80°C until analysis. Top and side
diameters and fiber length were used to calculate the volume of the fiber, assuming an
elliptical cross-section. The volume of the fiber per μl of loading buffer was then used to
standardize gel loads per unit fiber volume (ie, μl required to obtain 1.5 μm³ of fiber
volume). Because of this requirement for the loading volume (ie, 1.5 μm³ of fiber
volume), we selected a lower bound for fiber diameters of 60 μm to insure that there
would be enough sample to run triplicate analysis on each fiber. Thus, the fibers used in
these analyses do not reflect a random sample from each group.

For MHC protein measurements, samples were run on 4% acrylamide stacking/7.5%
acrylamide resolving gels at 70V for 3 hr and silver stained (Silver Snap; Pierce,
Rockford, IL). For actin protein measurements, samples from a sub-set of patients
(n=4/group) were run on 4-15% acrylamide/bis gradient gels at 150 V for 1.5 hr and
processed similar to MHC gels. Gels were scanned and the background-adjusted
brightness area product (BAP) of each MHC or actin band, run in triplicate, was
quantified (Quantity One; BioRad; Hercules, CA), as described. The BAP for each sample was then adjusted to the running internal standard. Samples in which no MHC band was apparent (density ≤ background) were given a value of zero.

**Single fiber mechanical measurements.** Aluminum t-clips were placed at both ends of the fiber and the fiber segment was mounted on hooks in dissecting solution at 20°C. Fibers were fixed at two points approximately 1 mm apart with glutaraldehyde, as described, with modifications. Briefly, fibers were placed in rigor solution (in mM: 134 potassium propionate, 10 imidazole, 7.5 EDTA and 2.5 EGTA; 20 2,3-butanedione monoxime at pH 6.8) and glutaraldehyde fixative (6% toluidine blue (w/v), 30% glycerol (v/v), 2% glutaraldehyde(v/v)) was applied (15 s/end) using the gravity feed method. The fiber was then placed in dissecting solution with 1% bovine serum albumin to absorb any remaining glutaraldehyde. Fibers were removed from the hooks, t-clipped in the fixed region, and the fiber material beyond the new t-clips removed.

**Ultrastructural measurements.** Shortly after obtaining tissue from the muscle biopsy, a bundle of muscle fibers was tied to a glass rod at slightly stretch length (~20% of initial bundle length) and was fixed in 1.5% glutaraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate and embedded in epoxy resin. The muscle bundle was cut in cross-section (~100 nm) and contrasted with lead and uranyl acetate prior to transmission electron microscopy. The number of thick and thin filaments per unit fiber cross-sectional area was assessed at 60,000X in 8 fibers per patient (Sterio Investigator v8.0; MBF Bioscience; Chicago, IL). Briefly, a measurement area was manually selected on the muscle fiber that contained clearly discernable thick and thin filaments. Counting frames (1 μm²) were automatically overlaid in a grid pattern and thick and thin filaments counted manually using standard rules. Data were expressed as the ratio of thick to thin
filaments. The muscle bundle was then cut 90° to the cross-sectional cut (~100 nm) and processed as above for electron microscopy (JEOL 1210 Transmission Electron Microscope; JEOL, Inc.; Peabody, MA). A-band (25 measurements/volunteer) and sarcomere length (50 measurements/volunteer) measurements were performed at 5,000X, while the percentage of fiber area occupied by myofibrils (3 images for 275 um²/volunteer) was performed at 8,000X, as described. All cross-sectional area, A-band length and sarcomere length measurements were made using NIH Image (Image J, National Institutes of Health, Bethesda, MD).

Protein and gene expression. Myofibrillar proteins were isolated from muscle tissue (~20 mg), as described above for tissue homogenates. All buffers were ATP free and contained 2 mM N-ethylmaleimide to inhibit proteasomal degradation and deubiquitinating isopeptidases. After protein content determination, samples were diluted in loading buffer and subjected to 4-12% acrylamide-bis gradient SDS-PAGE and routine Western blotting using specific monoclonal antibodies (slow MHC #MAB1628, 1:10,000; Millipore, Temecula, CA; ubiquitin #sc-8017, 1:2000; Santa Cruz Biotech, Santa Cruz, CA). After washing, blots were incubated with sheep anti-mouse IgG (#NA931, 1:2000, Amersham, Piscataway, NJ) conjugated to horseradish peroxidase for chemiluminescent detection (Pierce, Carlsbad, CA). We chose to assess MHC breakdown fragments using an antibody directed at the MHC I isoform because this is the most prevalent MHC isoform in human vastus lateralis muscle (Figure 1B). The pattern of MHC I breakdown fragments (Figure 5A) is similar to what has been previously noted in chymotrypsin digests of rat soleus muscle using this antibody, which likely reflects high molecular weight degradation fragments of myosin. Bands/portions of lanes were quantified by densitometry (Quantity One; BioRad; Hercules, CA). For MHC degradation fragments, the primary MHC band and the MHC
fragments were quantified separately. The densitometric signal for MHC degradation fragments was expressed relative to the primary immunoreactive MHC band to control for any variation in MHC protein content and isoform distribution among groups. To quantify the amount of ubiquitinated MHC, the ubiquitin signal corresponding to the MHC band was quantified and expressed relative to the total MHC band density determined from Simple Blue (Invitrogen, Carlsbad, CA) stained gels run concurrently.

For MHC and actin mRNA measurements, RNA was extracted from muscle tissue (≤8 mg) using the MELT Total Nucleic Acid Isolation System (Ambion, Austin TX). Multiplexed amplification reactions were performed using GAPDH as an endogenous control (Applied Biosystems, Assay ID: Hs99999905_m1) using the Quanta Perfecta QPCR Super Master Mix (Quanta Biosciences, Gaithersburg, MD). The following settings were used: Stage 1 (reverse transcription): 45°C for 5 min; Stage 2 (denaturation): 95°C for 3 min and Stage 3 (PCR): 95°C for 15 s and 60°C for 45 s for 40 cycles. The MHC I, IIA, IIX and actin oligonucleotides were purchased from Applied Biosystems (Assay ID: Hs01110632_m1; Hs00430042_m1; Hs00428600_m1 and Hs00559403_m1, respectively). For atrogene mRNA measurements, total RNA was extracted from muscle tissue (~25 mg) using Triazol reagent. Multiplexed amplification reactions were performed using 18S rRNA as an endogenous control (Applied Biosystems; Foster City, CA) using the TaqMan One step PCR Master Mix reagents kit (Applied Biosystems). The following settings were used: Stage 1 (reverse transcription): 48°C for 30 min; Stage 2 (denaturation): 95°C for 10 min and Stage 3 (PCR): 95°C for 15 s and 60°C for 60 s for 40 cycles. The MuRF-1 oligonucleotides were purchased from Applied Biosystems (Assay ID: Hs00261590). The sequences of the forward, reverse and double-labeled oligonucleotides for atrogin-1 were: forward 5'-CTT TCA ACA GAC TGG ACT TCT CGA -3'; reverse 5'-CAG CTC CAA CAG CCT TAC TAC GT-3'; TaqMan probe: 5'- FAM-TGC CAT CCT GGA TTC CAG AAG ATT CAA C-TAMRA-3'.
MHC and actin samples were run in duplicate and atrogene samples in triplicate. All fluorescence data were analyzed by SDS software (Applied Biosystems) and the threshold cycle (Ct) values for each reaction were used to calculate gene expression relative to controls, according to published algorithms (Applied Biosystems).

**Supplemental results:**

*Missing data.* Several data points are missing because of logistical and technical problems. For total body composition measurements, one heart failure patient was not tested because he exceeded the weight limit of the dual energy x-ray absorptiometry scanner. Because of this, peak VO₂ data for this subject are also excluded because they could not be corrected for fat-free mass. For computed tomography scans, one patient and one control did not have data because the scan files were corrupted upon transfer from the scanner computer to the storage database and in one control because logistical problems prevented the measurement from being completed. Data for peak VO₂ and accelerometry are not available on one subject because logistical problems prevented completion of these tests. For biochemical assessments (ie, protein and gene expression), variable sample sizes was due to limitations in tissue availability.

*Non-parametric statistical analysis.* Comparisons between heart failure patient and control groups using non-parametric statistical procedures (Mann-Whitney U test) did not alter the statistical significance of any of the differences noted within the body of the manuscript, as defined by parametric statistical procedures.

*Single fiber MHC and actin protein content.* In fibers used for MHC protein content measurements, actin bands were evident in all fibers studied, including those with MHC band density less than background. As the actin bands were not quantifiable on gels
used for MHC protein measurements, in a sub-set of patients (n=4/group) for which there were sufficient fibers for triplicate analysis, single fiber actin protein content was evaluated in the same fibers. We did not find differences in actin protein content between controls and patients (157 ± 7 vs. 149 ± 5 BAP x 10^3; n=51 and n=65, respectively).

**MHC degradation and gene expression.** MHC I breakdown fragments, the amount of ubiquitinated protein corresponding to MHC and expression of E3 ubiquitin ligases in a sub-sample of patients (n=4) and controls (n=4) are shown in Figure 5. We found no differences between heart failure patients and controls in the amount of MHC I breakdown fragments (Figure 5A,C) when assessing the densitometric signal of the fragments (C: 1671 ± 426 vs. HF: 1709 ± 362 arbitrary units (AU)) or when this signal was expressed relative to the primary immunoreactive MHC I band to account for MHC protein depletion in heart failure patients (C: 1.00 ± 0.21 vs. HF: 1.05 ± 0.24 AU). We also found no difference in breakdown fragment signal when expressed relative to total MHC protein determined from Simple Blue stained gels (C: 1.17 ± 0.26 vs. HF: 1.29 ± 0.34 AU). Similarly, there was no difference in the ubiquitin signal corresponding to intact MHC (Figure 5B,D; C: 1414 ± 44 vs. HF: 1301 ± 44 AU) or when the ubiquitin signal was expressed relative to total MHC protein determined from Simple Blue stained gels (C: 0.97 ± 0.30 vs. HF: 1.13 ± 0.07 AU). Parenethetically, in the non-myofibrillar protein fraction, there was no evidence for increased protein ubiquitination on an absolute basis (C: 1236 ± 71 vs. HF: 1268 ± 92 AU; representative gel in Supplemental Figure 1) or when expressed relative to the total MHC protein content determined from concurrently run Simple blue stained gels (C: 0.45 ± 0.01 vs. HF: 0.47 ± 0.03 AU), suggesting that there is no evidence for a general up-regulation of protein breakdown in heart failure patients. Finally, in a sub-set of volunteers (n=4 heart failure; n=6 controls; Figure 5E,F),
no differences were observed in the expression of the E3 ubiquitin ligases: MuRF-1 (C: 1.00 ± 0.20 vs. HF: 1.05 ± 0.20) or atrogin-1 (C: 1.00 ± 0.22 vs. HF: 1.00 ± 0.21).

Using 18S as a housekeeping gene, there was no difference in MHC I (C: 1.00 ± 0.12 vs. HF: 1.07 ± 0.20), MHC IIA (C: 1.00 ± 0.11 vs. HF: 1.02 ± 0.21), MHC IIX (C: 1.00 ± 0.43 vs. HF: 2.00 ± 0.75) or actin mRNA abundance (C: 1.00 ± 0.08 vs. HF: 1.03 ± 0.08).
REFERENCES


Supplemental Figure 1. Representative blot of ubiquitinated proteins in the non-myofibrillar fraction of skeletal muscle tissue homogenates in controls (C; n=4) and heart failure patients (HF; n=4).